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14. ABSTRACT

Bacillus anthracis is a gram positive, rod shaped and spore forming bacteria. It causes anthrax, a deadly human and animal disease that can kill its victims in three days. The spores of B. antrhacis can survive extreme environmental conditions for decades and germinate when exposed to proper conditions. Due to its potential as a bio-weapon, effective disinfectants that pose less harm to the environment and animals are urgently needed. Metal nanoparticles have the potential of killing microbial cells and spores. We present here the effect of Gold/Copper Sulphide core/shell (Au/CuS) nanoparticles on B. anthracis cells and spores. The results indicated that the continuous

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Bacillus anthracis is a gram positive, rod shaped and spore forming bacteria. It causes anthrax, a deadly human and animal disease that can kill its victims in three days. The spores of B. antrhacis can survive extreme environmental conditions for decades and germinate when exposed to proper conditions. Due to its potential as a bio-weapon, effective disinfectants that pose less harm to the environment and animals are urgently needed. Metal nanoparticles have the potential of killing microbial cells and spores. We present here the effect of Gold/Copper Sulphide core/shell (Au/CuS) nanoparticles on B. anthracis cells and spores. The results indicated that the continuous presence of 0.83 ?M during the spore growth in nutrient medium completely inhibited spore outgrowth. Au/CuS nanoparticles at concentration of 4.15 ?M completely inactivated B. anthracis cells (x 107) after 30 min of pre-treatment in any of the three buffers including water, PBS, and nutrient broth. However, the same and even higher concentrations of nanoparticles produce no significant spore (x 105) killing after 24 h of pre-treatment. SEM imaging, EDS analysis, and DNA extrusion experiments revealed that nanoparticles damaged the cell membrane causing DNA and cytosolic content efflux and eventually cell death. The study demonstrated the strong antimicrobial activity of Au/CuS nanoparticles to B. anthracis cells and revealed that Au/CuS NPs showed more effective inactivation effect against the cells than they did against the spores.

ANTIMICROBIAL EFFECTS OF GOLD/COPPER SULPHIDE (Au/CuS)
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CELLS

by

Ebenezer Addae

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To my wife, Bernice M. Addae and daughter Grace E. Addae: your continuous support has helped me through this project. May the Lord bless and help you in all you do.

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CHAPTER **I**

INTRODUCTION

Bacillusanthracis is a gram positive, rod shaped, non-motile and spore forming bacterium. Though it is believed to have been in existence in 1941 B.C., the description of the etiology of anthrax, the disease caused by *B. anthracis* by Robert Koch, marked the birth of medical microbiology in 1876 [1-3].Although anthrax is primarily a disease of herbivores, it affects other mammals also, including humans. *B.anthracis* enters its host through three main routes, namely cutaneous (through skin abrasions or skin lesions caused by biting insects), gastrointestinal (by the ingestion of spore contaminated food, water or forage) or pulmonary (by the inhalation of dust that contain spores). Infections via any of these routes can lead to fatal systemic anthrax. Upon entry, *B. anthracis*spores travel to lymph nodes where they germinate into vegetative bacilli. The vegetative bacilli produce the characteristic virulence factors: the toxin and the capsule -encoded for by two plasmids pX01 and pX02 respectively,and eventually enter into blood circulation. In the blood, the vegetative cells multiply rapidly and continue to produce the toxin until it eventually subdues the host system and causes a shock-like death. When bacilli from the dying or dead host are exposed to the air (oxygen), they sporulate and the cycle begins all over again[3-5].

Though its discovery formed the basis of the establishment of modern medical microbiology and vaccinology, *B.anthracis*has been used for many unethical purposes

since the early 1930s. For instance between 1932 and 1945, the Japanese Army's infamous Unit 731 experimentally infected prisoners with *B.anthracis*[3]. Also, in 1942, the British Army conducted an anthrax bioweapons test on the Gruinard Island in Scotland and subsequently prepared large amounts of cattle cakes infected with *B. anthracis* spores with the goal of spreading it on German agricultural land [3]. Even after the treaty on the ban on biologicalweapons was signed in March 1975, an anthrax outbreak infected 77 people killing 66 of them in Sverdlovsk, USSR (now Ekaterinburg, Russia) in April 1979.Soviet officials attributed this to the consumption of contaminated meat; however, western governments believe the accidental releaseof *B.anthracis*spores froma Russian bioweapons production research facility was the cause [3, 6]. The most recentunethicaluse of *B. anthracis* was the 2001 bioterrorism attacks in the United States of America. In this incident, terrorists mailedatleast five letters containing dry *B. anthracis* spores to people in the news media and government leading to the death of five people.About 30,000 people were treated with antibiotics and numerous public buildings were decontaminated in response to this attack [7, 8].According to the FBI (Federal Bureau of Investigations), the attack cost over one billion dollars in damages with cleanup cost contributing about three hundred and twenty million dollars [8, 9] .

While great strides have been made in theprevention and treatment of natural anthrax infections, the battle against *B.anthracis*used as a bioweapon will likely continue; especially considering the fact that no effective inhibitory agent has been found for the spores of *B. anthracis*. According to Whitney et al. there is insufficient scientific knowledge on the decontamination of buildings from the intentional release of *B.*

anthracis spores [10]. After the 2001 attack, federal agencies used chlorine dioxide gas, vaporized hydrogen peroxide, para-formaldehyde and gamma radiation to decontaminate the buildings affected[9, 10]. However, these antimicrobial agents are costly and may themselves pose harm to first responders. For instance formaldehyde is known to play a possible role in carcinogenesis [10]. An additional factor that leads to increased cost is the need for buildings to be completely evacuated for the duration of decontamination (because of the potential harm these chemicals can cause to humans). This leads to the loss of labor time. In addition, most of the data available on sporicidal agents is based on spores of bacillus species other than *B. anthracis*. Since the 2001 bioterrorism attacks, increased attention has been paid to finding efficient, cost effective ways to decontaminate environments inhabited by *B. anthracis* spores and cells. This is necessary to protect first responders and the army against occupational related infections and to reduce the cost of decontaminating buildings when accidentally occupied by *B. anthracis* spores[9, 10].

Various nanoparticles (NPs) have distinct physical and chemical properties and have gained increased attention in biological, biomedical, physical and pharmaceutical applications. Studies have shown that antimicrobial formulations made from NPs have high bacteriocidal activity [11, 12]. Klabunde and co-workers recently reported that dry powder formulations and slurries of magnesium oxide NPs have great biocidal effect on both gram positive and gram negative bacteria and their spores [13]. Sondi and Salopek-Sondi also reported that, silver NPs penetrated the cell membrane of *E. coli* cells and killed them[12]. Zawrah and El-Moez investigated the antimicrobial activity of Au NPs on various food borne pathogens and reported that drugs coated with NPs were highly

effective against *C. albicans, A. niger and A. flavus*and that the Au NPs minimized treatment durations and side effects of the drugs[14]. Generally, bacterial cells can be destroyed in three main ways: inhibition of DNA synthesis and integrity, inhibition of transcription and translation, and inhibition of cell wall synthesis and integrity [15].The mechanism of metal NPs involves destruction of cell wall or membrane integrity[16-18]. The negatively charged bacterialcells are believed to cultivate electrostatic interactions between the cells and the positively charged NPs resulting in the compromise of the cell membranes and eventually, cell death[19].Based on the potential of metal and other NPs as biocides, we postulated the possibility of using Au/CuSNPs as a biocidal agent against *B. anthracis* spores and cells.

The first synthesis of Au/CuS core shell NPs was reported by Sun et al.[20]. Au/CuS core shell NPs combine the surface plasmon resonance coupling of Au NPs with the ease of preparation, low cost and the small size of CuSNPs for photothermal therapy in cancer treatment. However, there is no report on its antimicrobial activity. The Au/CuS core/shell NPs being used in this research was produced by collaborators from the Physics Department of the University of Texas[21].

The objective of this research was to test the antimicrobial effects of Au/CuS NPs on *B. anthracis* spores and cells. The four specific aims were

1. To examine the physical properties of the Au/CuS NPs using transmission electron microscopy (TEM) and Energy-dispersive X-ray spectroscopy (EDS).

2. To investigate whether Au/CuSNPs could effectively inactivate *B. anthracis* spores and cells considering variables such as the Au/CuS concentration, treatment medium, and treatment time.

3. To study the interactions between Au/CuS and *B. anthracis*cells and spores using fluorescence microscopy, scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy(EDS).

4. To investigate the possible antimicrobial mechanism of Au/CuS on *B. anthracis*cells and spores.

CHAPTER **II**

LITERATURE REVIEW

2.1 *Bacillus anthracis*

Bacillus anthracis is a gram positive, rod shaped, aerobic, non-motile and spore-forming bacteria that mainly affect herbivores [22]. They are 1-1.1 μm wide and 5-6 μm long and grow as long square ended chains (Figure 2.1A). When exposed to oxygen under limited CO_2 conditions, *B. anthracis* cells form spores at temperatures 14-42 ^{o}C (optimum 21-37 ^{o}C). It takes about 48 h for the oval shaped spores to mature and they are released after lysis of the bacterium (Figure 2.1B) [5].
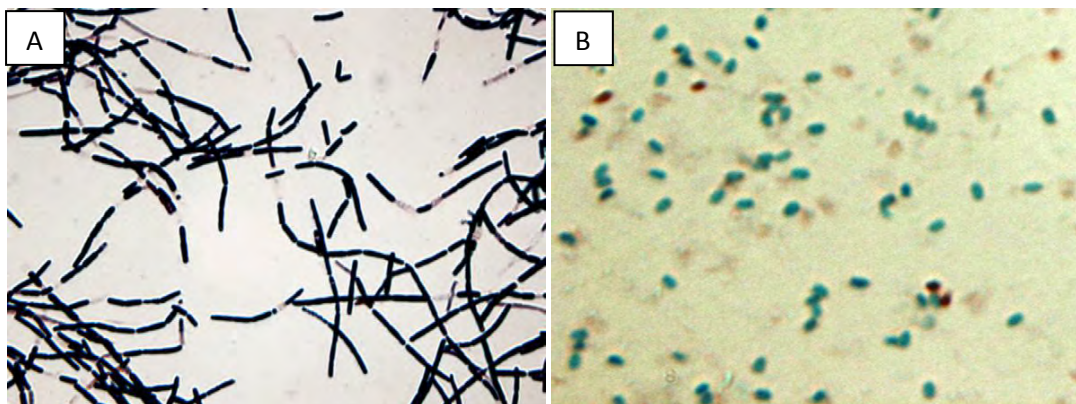
Figure 2.1 Microscopic images of *B. anthracis*cells and spores: (A) is vegetative form, gram stain; (B) is spore form Shöeffer Fulton stain. (reprinted from[5]).

B. anthracis grows well on ordinary medium at pH 7-7.4 and temperatures 12 -42 ^{o}C, with optimum temperature at 37 ^{o}C. It forms white colonies with rough "glass bead" surfaces 3-4 mm in diameter on nutrient agar. On tryptose broth, it flocculates and eventually settles to the bottom leaving a clear liquid above. It does not cause hemolysis on blood agar as observed in other bacteria [5].

2.2 The Spores of *B. anthracis*

B. anthracis forms spores in response to starvation - insufficient nutrients and/or CO_2. The spores have a highly ordered structure with a multilayered proteinaceous shell called the coat. The coat is responsible for resistance and germination of the spore. It provides mechanical integrity, excludes large toxic molecules and simultaneously allows small nutrient molecules to penetrate and interact with germination receptors [23]. In addition to the protective coat, *B. anthracis* spore is encased by another layer of structure called the exosporium consisting of a paracrystalline basal layer and a hair-like outer layer (Figure 2.2). The exosporium and the coat are structurally and biochemically distinct. According to Liu et al [24], spores that do not have the exosporium are as infectious as those that do have them so the exosporium may not contribute significantly to infection. However, theexosporium may contain important antigens and markers that aid in diagnosis [24]. The exosporium is composed of lipids, proteins and carbohydrates. Mature spores are metabolically inactive and are resistant to temperature extremes, physical damage, desiccation and harsh chemicals. This allows the spores to survive for several decades until they encounter a favorable nutrient rich environment

wheretheygerminate. The ability of spores to survive harsh environments for decades contributes to their ability to be formulated and dispensed as biological weapons [3, 5, 24].
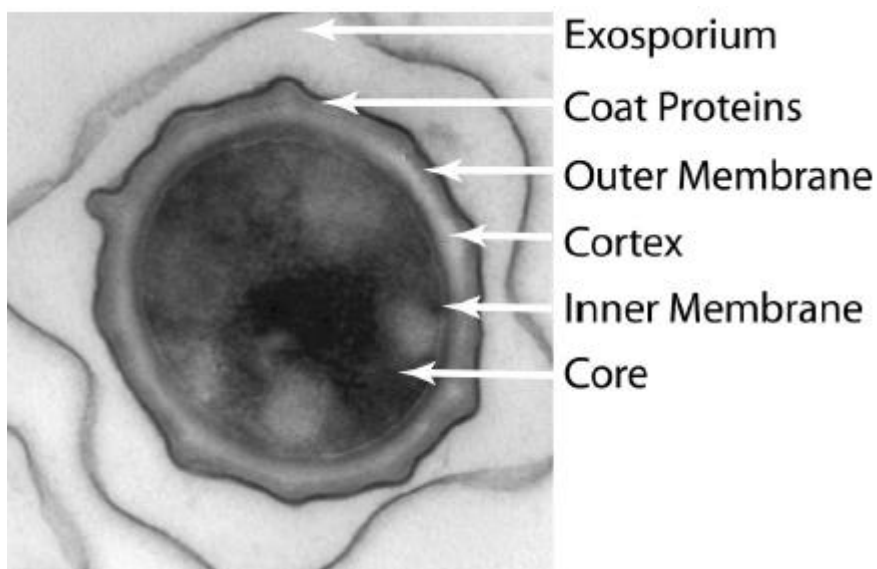
Figure 2.2:TEM of *B. anthracis* **endospore: Transmission electron micrograph of negatively stained (2% uranyl acetate)** *B. anthracis* **endospore. Magnification: X 92,000. (reprinted from[24])**

2.3 Anthrax disease in humans

Anthrax, the disease caused by *B. anthracis* rarely affects humans. However, people who come in contact with farm animals and contaminated animal products can be infected by the disease. There are three main forms of the anthrax disease in humans defined by the mode of infection: cutaneous, gastrointestinal or inhalational anthrax.

Cutaneous anthrax occurs when the spores of *B. anthracis* enter skin abrasions and cuts or a biting fly transmits the spores to the skin. This leads to a painless ulcer that usually

resolves without major consequences. About 20% of patients develop septicemia and die when cutaneous anthrax is untreated [3, 25].

Gastrointestinal infections occur by the ingestion of undercooked meat contaminated with *B. anthracis* spores. It is present in two clinical forms namely abdominal and orophageal anthrax. Symptoms of initial abdominal anthrax include nausea, vomiting and fever. Progressive abdominal anthrax results in severe abdominal pain and bloody diarrhea, followed by septicemia. Clinical manifestations of orophageal anthrax include fever, cervical lymphadenopathy, oedema, dysphagia and sore throat. Gastrointestinal anthrax is rare but fatal, leading to death within 2-5 days. However, if diagnosed early, it can be cured by the administration of antibiotics [3, 25].

The third form of the disease is inhalational anthrax, resulting from the inhalation of aerosolized *B. anthracis* spores. This is the most dangerous form of the disease. Until the 2001 bioterrorism attack, when letters were deliberately contaminated with *B. anthracis* spores, the spread of inhalational anthrax was limited to industrial exposure; mainly in the tanning and textile industries [25, 26]. Once in the body, spores are transported to the lymph nodes by macrophages where they germinate into vegetative bacilli. The bacilli then produce the two principal virulence factors, toxins and capsules, and continue to spread to other nodes and eventually to the blood stream. In the blood, the cells continue to reproduce and produce the toxin, eventually killing its host through shock [3]. Symptoms of inhalational anthrax include fever, rapid and faint pulses, cyanosis, tachycardia and pleural effusion [3].

2.4 Major virulence factors

Wild type strains of *B. anthracis* carry two plasmids, pX01 and pX02 which carry the genes responsible for the virulence factors. PX01 carries the genes that encode the toxins and pX02 carries the genes that encode the biosynthesis of the capsule [3, 5, 27]. The complete plasmid DNA sequence of pX01 is available on GenBank with accession numbers NC_001496.1, NC_012579.1 and CP001216.1 and that of pX02 has accession numbers NC_012577.1 and CP001214.1.

The pX01 encodes three proteins: the oedema factor (EF), the lethal factor (LF) and the protective antigen (PA) which combines to give the two toxins of anthrax. The protective antigen has no direct toxic effect but acts as a transporter of the LF and the EF into the cell. It first binds to the tumor endothelium marker 8 (TEM 8) and the capillary morphogenesis 2 (CMG2), both of which are cell surface receptors expressed in many cell types including cells of the immune system[26]. Under acidic cell conditions, the C-terminal domain of the PA is released and it oligomerizes into truncated heptamers $(PA_{63})_7$; simultaneously associating with lipid rafts (Figure 2.3). The raft-PA_7-EF/LF complex is then internalized through endocytosis. The acidic environment of the endosome causes the PA to rearrange, forming a channel in its membrane. This channel allows the transport of the EF and LF into the cytosol of the cell (see Figure 2.3) [28]. In the cytosol, the EF, which is a calcium calmodulin dependent adenylatecyclase, causes a dramatic increase in the cytosolic cAMP leading to an imbalance of water homeostasis and eventually cell death. This water imbalance is responsible for the oedema symptoms

observed in individuals affected with anthrax. The LF on the other hand is a Zn^{2+} metalloprotease that cleaves most isoforms of the mitogen activated protein kinase (MAPK) kinases (MEKs). MEKs are part of a major phosphorelay (signaling) pathway that links surface receptors to the transcription of several genes in the nucleus. Hence, cleavage of MEKs by LF leads to the lack of transcription and the subsequent synthesis of the affected proteins.

The capsule encoded by the pX02 is a linear polymer of D-Glutamic acid. It enables the bacteria to resist phagocytosis by macrophages and hence evade the host immune system.
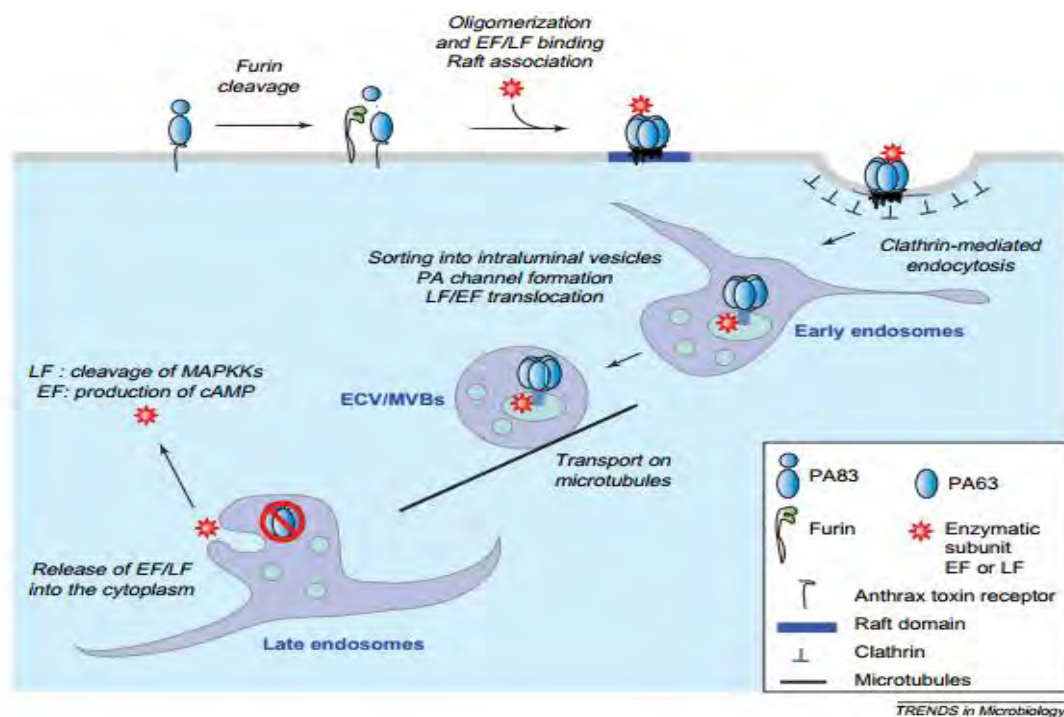
Figure 2.3: Anthrax toxinand the endocytotic pathway in mammalian cells. (Reprinted from [28])

2.5 Methods for inactivating *B. anthracis*spores and cells

A number of approaches have been established for inactivating the spores and cells of *B. anthracis*. These include the use of:

- Chemical inactivation

- Heat inactivation

- Radiation application

- Carbon nanotube and other NPs

- Combinatory treatment method

2.5.1 Chemical inactivation

Chemical inactivation of *B. anthracis* spores is the most popularly used method. Formaldehyde solution or gas has been used as a disinfectant since the late 1980s. *B. anthracis* spores (x 10^5cfu/mL) were successfully inhibited by 4% formaldehyde in water. Controlled experiments testing the effect of formaldehyde gas on *B. globigii* revealed that humidity greatly affects its activity [10]. Other chemicals used to inactivate*B. anthracis* spores include sodium hydroxide, paracetic acid, glutaraldehyde, hydrogen peroxide, sodium hypochlorite and free available chlorine. Table 2.1 below summarizes the concentrations and treatment conditions for inactivating various spore concentrations.

Table 2.1: Chemical inactivation of *B. anthracis* spores

Chemical	Concentration	Inoculum size	Reference
Calcium hypochlorite	20mg/L pH 8.0, 20 oC	3-4 x10^5cfu/ml of *B. suttilis*.	[29]
Free available chlorine	2.3-2.4 mg/L pH 7.2.0, 22 oC	1.1 x 10^5cfu/mL of *B. anthracis*	[30]
Sodium hypochlorite	0.05%, pH 7 or 20 oC	1.6 – 2.2 x10^9cfu/mL	[31]
Hydrogen peroxide	0.88 mol/L, pH 5.0	10^6cfu/ml *B. suttilis*	[32]
Peracetic acid	0.13 mol/L, pH 5.0, 6.5, 8.0	10^6cfu/ml *B. suttilis*	[33]
Glutaraldehyde	2% in water, pH 8.0	10^8cfu/mL spores *B. anthracis*	[34]
Sodium hydroxide	5%, 27.8 or 21.1 Oc	7 x 10^9 spores/ml *B. suttilis*	[35]

2.5.2 Heat inactivation

Moist heat, dry heat or boiling can be employed to inactivate bacterial spores and cells. It has been reported that the concentration of *B. anthracis* spores can be reduced by 10^6cfu/mL by heating with moist heat for 5-15 minutes at 120 ^{o}C, 5-10 minutes at 105 ^{o}C or 10 minutes at 100 ^{o}C, or 20 min at 90 ^{o}C. Also, boiling *B. anthracis* spores at 3 x 10^8 and 7.5 x 10^8 for 10 and 5 min, respectively, completely inactivated them [10, 36-38]. However, the thermal kinetics of inactivation in a buffered is not representative of their inactivation in food. Xu et al. (2006) investigated the inactivation of *B. anthracis* in milk. They found that, though standard pasteurization (72 ^{o}C for 15 s) could not inactivate high concentrations of anthrax spores in milk, ultra-high pasteurization at 120 ^{o}C for 6-10 s or boiling for 4 minutes inactivated spores in both whole and skimmed milk. Also, ultrahigh

pasteurization produced no significant degradation of milk as compared to standard pasteurization[36, 37].

Frances A. (1956) investigated different temperature and time conditions and found that, $6 \times 10^3 - 1.2 \times 10^4$cfu/mL *B. anthracis* spores were inactivated when treated with dry heat at 140 ^{o}C for greater than 90 min. Other temperature and time conditions found to inactivate spores included 150-160 ^{o}C for 10 min, 180 ^{o}C for 2 min and 200 ^{o}C for 30 s[38].

2.5.3 Radiation application

Horne et al. (1959) reported that, 1.5 megarads from a 200,000 rad/h cobalt source could effectively kill most resistant spores in contaminated goat's hair. In relation to that, gamma radiation was used to disinfect goat's hair contaminated with *B. anthracis* spores in the 1950s and 1960s. Also, gamma radiation was used to decontaminate mails from affected buildings after the 2001 bioterrorism attack [10, 39].

Ultra violet (UV)light is another form of radiation tested as an antibacterial agent. It is particularly useful in killing airborne bacteria. Studies by Dietz (1980) reported that a high dose of UV radiation was needed to inactivate *B. anthracis* spores [40]. Previous reports involving spore forming bacteria (*B. subtilis and B. megatherium*) also indicated that twice as much UV light was required to kill spores as was needed for cells. However, Sharp (1939) reported that 452 ergs/mm^2 of UV light was required to destroy a mixture

of *B. anthracis*spores and cells but that this mixture was not twice as resistant to UV light

as were*E. coli* cells [41-43].

2.6 Antimicrobial activity of NPs

NPs are gaining increased attention for use as antimicrobial agents. Sloan and Farnsworth

(2006) tested the *in-vitro* antibacterial activity of various metal NPs against *E. coli and B.*

anthracis. In their work, they exposed *B. anthracis*spores to silver, silver-iron and silver

nickel NPs of different sizes in suspension and then plated the bacteria. They found that

the NP concentrations at 100 and 500 µg/mL killed *B. anthracis* spores in a size

dependent manner. In other tests, Sondi and Sondi(2004) investigated the antimicrobial

effects of silver NPs on *E. coli* cells by supplementing LB agar plates with 10 − 100

µg/cm^3 silver NPs and culturing *E. coli* cells (10^5 CFU) on them. Their findings indicated

that, 10 µg/cm^3 silver NPs inhibited bacteria growth by 70% and that 50-60 µg/cm^3

caused a 100% inhibition of bacterial growth. They also performed other tests where the

bacterial growth rate in liquid LB medium was measured by optical density readings at

600 nm. They found that, 10, 50, and 100 µg/cm^3 silver NPs slowed growth of 10^7*E. coli*

cells in a concentration dependent manner [44].

2.7 Possible mechanisms of NP's antimicrobial activity

Many explanations of the mechanism by which NPs destroy cells and spores have been

offered. Generally, bacterial cells can be destroyed in three main ways: Inhibition of

DNA synthesis and integrity, inhibition of transcription and translation and inhibition of

cell wall synthesis and integrity[15]. Themechanism most commonly reported for NP antimicrobial activity involved the destruction of cell wall or membrane integrity[16-18].

According to Sondi and Sondi (2004), silver NPs interacted with the cell membranes of *E. coli* cells and caused structural changes, degradation and cell death. Sloan and Farnsworth [45] also explained that when *B. anthracis* spores were exposed to silver, silver nickel and silver-ion NPs, the NPs attached to the spores but were ineffective in killing the dormant spores. However, after the spores were transferred to blood agar plates and germinated and grown into cells, the NPs neutralized the bacterial cells[45]. Cell membranes of bacteria are believed to be negatively charged and these negative charges foster electrostatic interactions between the cells and the positively charged NPs resulting in the eventual destruction of the cell membranes.

2.8 Gold NPs and its antimicrobial activity

Gold, being one of the first metals to be discovered, has been extensively studied for various applications. The synthesis of gold NPs of varying desirable properties (such as size, optical properties and shape) has been made possible by the improvements in technology. Functionalized gold NPs have applications in drug delivery, biomedical imaging, cancer therapy, biosensor technology, immunoassay and water purification [46]. Theuse of gold NPs has generated much interest for the following reasons:

1. There is a long history of the medicinal application of gold. Chinese, Arabic and Indian scientist used colloidal gold for medicinal purposes in the fourth and fifth

centuries and in recent times, colloidal gold was proposed to ease the suffering of inoperable cancer patients [46].

2. Gold NPs can be synthesized in several ways that are comparatively simple, safe, cheap and reliable.

3. Different sizes and shapes of gold NPs can be synthesized by simply modifying reaction parameters

4. The surface of gold NPs can be modified because of their highly reactive nature and their ability to interact with amine and thiol containing molecules such as proteins, DNA and enzymes. Reports on the conjugation of gold NPs with various biomolecules are available.

5. Gold NPs have unique optical and electronic behaviors.

6. Gold NPs are nontoxic and biocompatible [47].

Several articles have recently reported on the applications of gold NPs. Lima et al. reported in 2013 that gold NPs are effective as antimicrobial agents against *Escherichia coli and salmonella typhi*[48]. Lokina and Narayanan also recently investigated the antimicrobial effects of gold NPs against *Staphylococcus aureus, citrobacterkoseri, Bacillus cereus, Pseudomonas aemgiosa and Escherichia coli.* They reported that, the gold NPs (MIC = 0.3125 mg/mL) showed strong antimicrobial effects against *B. cereus* which is a surrogate strain for *B. anthracis*[49]. These same NPs show good anticancer activity against Hela cell lines.

2.9 Gold/Copper Sulphide NPs

Even though gold NPs have had great success in photothermal use because of their rich surface chemistry and ease of synthesis, they have limitations: the surface Plasmon Resonance of gold NPs is dependent on the dielectric constant of its environment. However, the high reactivity of gold NPs allows for their conjugation with various chemical agents. CuS is a semiconductor that is inexpensive, has low cytotoxicity and has an intrinsic near infrared absorbance (NIR) that originates from the d-d transition of Cu^{2+} ions. Li et al.[51] developed CuS NPs for the photothermal ablation of cancer cells. However, their low photothermal conversion property and the higher power intensity required for their use (16 and 24 w/cm^3) – 48 and 72 times higher than the safety of laser intensity for human skin exposure - has limited their applications.

A combination of gold NPs and CuS NPs would produce a new NP (Au/CuS) that combines their individual advantages for photothermal applications. Copper sulphide has the added advantage of being less expensive than gold, hence reducing the overall cost of the NP [50, 51]. In 2011, Lakshmanan[50] designed Au/CuS core/shell structure to overcome the limitation of the current NP system for photothermal therapy. These NPs can be used for *invivo* applications by taking advantage of the d-d transition peaks of CuS without worrying about the Plasmon absorption shifts of gold NPs. Also, they have higher absorption intensities than the Au or CuS alone and hence have improved photothermal conversion efficacy. Lakshmanan also demonstrated the uptake of these

NPs by cancer cells, concluding that they have high potential for use as photothermal ablation agents. More information about Au/CuS NPs is available from[21, 50].

In this study, we investigated the bacteriocidal effects of the recently synthesized Au/CuS NPs. To our knowledge, this is the first report of work done on the antimicrobial effects of Au/CuS core/shell NPs.

CHAPTER **III**

MATERIALS AND METHODS

3.1 Media Preparation

Nutrient broth (Becton, Dickson and Company, Sparks, MD) was used for bacterial cell growth. To prepare the nutrient broth, 8 g of nutrient broth was completely dissolved in 1 L of deionized (DI) water. When necessary, the dissolution was aided by boiling of the medium. After cooling down to room temperature, the pH was adjusted to 6.8 using a SevenMulti pH meter (Mettler-Toledo GmbH, Switzerland). The medium was then autoclaved at 121 °C for 20 min and stored at 4 °C for further experimental use.

The Difco sporulation medium (DSM) was used for growing *B. anthracis* spores. DSM was prepared according to the method described by Nicholson and Setlow (1990). To make 1 Lof medium, 8 g of Difco nutrient broth (Becton Dickson and Company, Sparks MD) was dissolved in DIwater and 10 mL each of 10% (w/v) potassium chloride (KCl) and 1.2 % (w/v) magnesium sulfate ($MgSO_4$) added and the volume brought to 1 Lwith distilled water. The pH was then adjusted to 7.6 using 0.1 M NaOHor 0.1 M HCl and the medium was autoclaved at 121 °C for 20 minutes using the SR-24C sterilizer (Consolidated Stills and Sterilizers, Boston, MA). Prior to use, 1ml each of 1 M calcium nitrate ($Ca(NO_3)_2$), 0.01 M manganese chloride ($MnCl_2$) and 1 mM ferrous sulfate ($FeSO_4$) was added to the 1L preparation. The volume of the $Ca(NO_3)_2$, $MnCl_2$ and $FeSO_4$ was adjusted depending on the volume of the medium needed at a time.

Luria Bertani (LB) Agar was used for enumerating viable bacteria or spore numbers using the traditional surface plating method. In making the LB agar medium, 40 g of LB Agar Miller from Fisher Bioreagents was dissolved in 1 L of DIwater and the pH was adjusted to 7.0. The medium was autoclaved at 121°C for 20 min. When the medium was cooled down to about 60-70 ˚C, about 20 ml was poured into each sterile 100 mm X 15 mm polystyrene petri dish (Fisher Scientific, Pittsburgh, PA). The plates were allowed to set and packed into sterile plastic bags, and stored at 4°C for further use. Prior to use, the plates were opened and any moisture collected allowed toevaporate in a level II biosafety cabinet.

3.2 Phosphate Buffered Saline (PBS) Preparation

PBS was prepared using PBS powder packets obtained from G-biosciences (St. Louis MO). To prepare 0.1 M (10X) PBS solution with pH 6.9 – 7.2, 1 packet of PBS powder was dissolved into 1 L of DIwater. The solution was then autoclaved at 121 ^{o}C for 20 min and stored at room temperature for further use.

3.3 Preparation of *B. anthracis*Cells

B. anthracis cells were grown by inoculating 50 mL of nutrient broth (pre-warmed to 37 ^{o}C) with 200 μL of *B. anthracis* vaccine Sterne 34F2 from Colorado vaccine company (Denver, CO). The culture was incubated at 37 ^{o}C with 250 rpm agitation in Excella E25 shaker incubator New Brunswick Scientific (Edison, NJ) for 18-20 h. The cells were

centrifuged at 4000 xg using a Beckman Coulter Avanti J26XP centrifuge (Atlanta GA) and the supernatant was discarded. The cell pellet was washed three times with desired buffers (sterile DIwater or nutrient broth or PBS). The cells were resuspended in respective buffers, and diluted to desired concentrations for further experimental use. Cell concentration was determined by the traditional surface plating method described below. Freshly prepared cells were used for each experiment[52].

3.4 Preparation of *B. anthracis*Spores

To prepare spores, *B. anthracis* cells were grown by inoculating 100 µL of *B. anthracis* vaccine Sterne 34F2 into 25 ml DSM and incubating at 37 ^{o}C with 150 rpm in the Excella E25 shaker incubator until they reached mid-log phase, $0.45<A600<0.6$. The culture was then diluted 1:10 into 225 ml DSM to obtain a total volume of 250 ml in a 2 L flask. The culture was incubated further at 37 ^{o}C and 150 rpm for 48 or more hours. During incubation, drops of the culture were placed on a glass slide and examined under the microscope for spore formation, until approximately >95% free spores were obtained. To purify the spores, the culture was treated at 70 ^{o}C for 25 min to kill vegetative cells and centrifuged at 8000 xg for 5 min. The supernatant was discarded. The pellet was washed six to eight times with cold DIwater to get rid of cell debris. After the final wash, the spores were re-suspended in the cold distilled water and stored at 4 ^{o}C for future use Prior to each use, the spore suspension was incubated in a 70 ^{o}C water bath for 20 min and washed twice with cold water, and resuspended in appropriate medium [52]. The spore concentration was determined using a hemocytometer.

3.5 Determination of Spore Concentration in Suspensions Using

Hemocytometer

Spore concentration in the suspensions was determined using ahemocytometer as described by P. J. Hansen of the University of Florida [53]. The spore suspension was diluted appropriately, and the diluted sample was added to the hemocytometer grid using a pipette. The concentration of spores was determined by counting the number of spores in five squares in the large middle square (indicated "R" in Figure 3.1 below) under the microscope (Nikon Eclipse E600FN, Japan). The spore concentration was calculated as follows:

Cell concentration per milliliter = Total spore count in 5 squares x 50,000 x dilution factor.
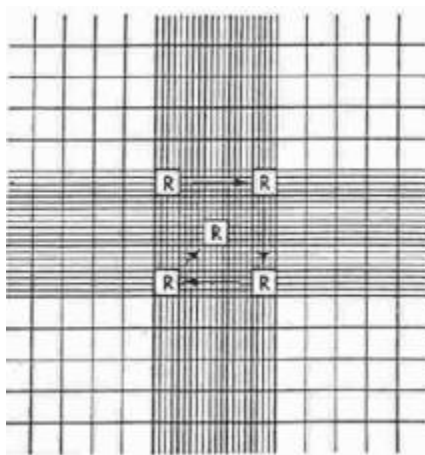

Figure 3.1: Counting grid on the hemocytometer[53].

3.6 Au/CuS Nanoparticle treatment to *B. anthracis* Cells and Spores

Au/CuS nanoparticles (NPs) were obtained from the Physics Department of the University of Texas, Arlington. The nanoparticles were in distilled water suspension at a concentration of 83 μM Au and 300 μMCuS. This Au concentration was used to make all calculations for this research. According to Lakshmaman et al (2011), the nanoparticles were made by dissolving gold nanopolyhedra in nickel thiobenzoate and then adding copper nitrate solution and heating at $140^{o}C$ for 3 h [50].

To treat the *B. anthracis* cells or spores with Au/CuS NPs, 900 μL of the purified *B. anthracis* cells/spores (OD_{600}nm = 0.9-1; 10^5 spores and 10^7 cells) was placed in 1.7mLeppendorf tubes and added 0, 1, 10 or 50 μL of the Au/CuS NPs to reach the final concentrations of 0, 0.083, 0.83 and 4.15 μM. Respective buffer (DI water or nutrient broth or 10X PBS) was added so that the total volume in each treatment was 1 mL. The cells or spores added in their respective buffers were used as controls. The samples were rotated at 15 rpm on the Dynal Biotech Rotator (Lake Success, NY) for 0.5, 1, 3, 6, 12 and 24 h at room temperature.

3.7 Monitoring Cell or Spore Growth by Optical Density (OD) Measurement

The effect of the NPs on the inactivation of *B. anthracis* cells or spores was evaluated by examining the cell or spore growth after they were treated by NPs comparedto the controls. Cell or spore growth was monitored by OD measurement at 600 nm. Specifically, the NP treated cell or spore samples (1 mL) were transferred to 9 mL of

nutrient broth. The 10 ml samples were then incubated at 37 $^{\circ}$Cat 250 rpm in the Excella E25 shaker incubator. OD_{600} nm was read every 30 min for the first three h and then every hour for a maximum of 10 h using aSpectra Max Plus 384 spectrophotometer (Molecular Devices, Synnyvale CA). The growth curve for each sample was generated by plotting the OD_{600} nm value against the growth time. To evaluate growth of the treated samples, their growth curves were compared to that of the controls by comparing the time pointsat which growth reached exponential phase.

3.8 Determination of cell or spore concentration using traditional surface plating method.

The inactivating effect of the NPs on *B. anthracis* cells or spores was also evaluated by the reduction in the number of viable cells or spores following the treatments. The viable spore and cell concentrations in treated samples and control samples were determined using the traditional surface plating method.

Specifically, after treatment serial dilutions (1: 10) of the treated samples (spores/cells and nanoparticle mixture) and the control samples (spores/cells without nanoparticles) were prepared. 100 µL of the appropriate dilution of the samples were surface plated on LB agar plates. The plates were allowed to dry in a level II biosafety cabinet and then incubated overnight at 37 $^{\circ}$C in a Fisher Scientific Isotemp Incubator (Dubuque, Iowa). The number of colonies was counted and the number of cells/mL in the original preparations was calculated as colony-forming unit per milliliter (CFU/ml). At least three

replicates of each treatment were plated and all preparations were performed in a level II biosafety cabinet.

3.9 Fluorescence and Scanning Electron Microscope (SEM) Imaging and EDS

The purpose of imaging was to investigate the interactions of *B. anthracis* cells or spores with Au/CuS nanoparticles. Fluorescence microscope images were obtained after cells had been treated with 0.83 μM Au/CuS for 10, 20 and 30 min and stained with Live/dead BacLight bacteria staining kit (Invitrogen, New York) for 15 min. According to the manufacturer's instructions, a 1:1 mixture of the live (syto 9) and dead (PI) stains was prepared and 3 μL added to 1ml of each of the samples (*B. anthracis* cells and cells treated with nanoparticles). Five μLof each of the stained samples was placed on a microscope slide and covered with the cover slip. The images were obtained using a Nikon Eclipse Ti-FLC-E confocal microscope (Japan) at X10 magnification.

For the SEM imaging, the spores or cells were first treated with 0, 0.83 and 4.15 μM Au/CuS nanoparticles for 30 minutes and fixed in bacterial fixative (4% Formaldehyde and 2% Glutaraldehyde in 1x PBS). FiveμL of the fixed samples was placed on a cover slide and air dried. The dried sample was then coated with gold using Denton Vacuum Desk IV (Czech Republic) with a gold target and the SEM images were taken using the FEI XL30 microscope (Netherlands) at the Shared Materials and Instrumentations Facility at Duke University [54]. Prior to coating with gold, the elemental composition of

the samples was obtained by EDS analysis using FEI XL30 ESEM with XFlash 4010 EDS Detector (Czech Republic).

3.10 Transmission Electron Microscope Imaging of Au/CuS Nanoparticles

The NPs were diluted 1:1, 1:10 and 1:100 in DI-H_2O to obtain 8.3, 0.83 and 0.083 µM concentrations and 5 µL drops of each dilution was placed on a cover slip and air dried on a 2 mm X 2 mm cover slip. FEI Tecnai G2 Twin with a LaB6 filament (Czech Republic) was used to image the nanoparticles.

3.11 Measurement of the Efflux of Cytoplasmic DNA

Possible cellular membrane damage caused by NPs treatment was examined by measuring intracellular material in the solution of *B. anthracis*cells exposed to Au/CuS NPs. The concentrations of DNA in solutions for cells incubated with and without Au/CuS was determined using the method described by Kang et al. (2007) [55] with slight modifications. After cells had been treated with 0, 0.083, 0.83 and 4.15 µM Au/CuS, the treatment was centrifuged at 10,000 xg for 4 min and the supernatant was filtered through 0.2 µm Millipore filter to eliminate residuals. Ninety-nine micro literaliquots of the filtered samples were put in black 96-well assay plates (Corning Incorporated, NY) and 1 µL of 0.5 mg/ml 4, 6-diamidino-2-phenylindole (DAPI) was added to obtain a final DAPI concentration of 5 µg/mL in each well. The plate was then covered with aluminum foil to keep it from light and incubated at 4°C for 2 h. DAPI stain

has strong binding affinity for the A-T region of DNA and the intensity of its fluorescence is proportional to DNA concentration. Emission fluorescence at 470 nm was measured after excitation at 370 nm. Salmon sperm DNA (Amresco, Solon, Ohio) was used to generate the calibration curves [55, 56] to quantify the amount of DNA of the NPs treated samples.

3.12 Data analysis

Each experiment was repeated three or more times with each having three or more replicates. The mean and standard deviation of each replicate set was determined and used to plot figures.

CHAPTER **IV**

RESULTS AND DISCUSSION

4.1 Properties of Au/CuS NPs

We first examined the Au/CuS NPs received from the University of Texas at Arlington using TEM. Figure 4.1 shows the TEM images of the Au/CuS NPs (at 1:10 dilution of the received sample). Two main particle sizes were observed:

1. Smaller sizes: 2-5nm, indicated to by the black arrows
2. Larger sizes 10-20nm; indicated by the red arrows

The smaller sized NPs (2-5 nm) dominated and a few larger sized NPs were observed mainly in the higher NP concentrations (1:1 and 1:10 dilutions).

The larger particles observed could be clumps of the NPs or impurities introduced during the dilution, drying, or transportation process. However, since these were observed mainly in the higher concentrations of the NPs, they are likely to be clumps of the NPs. The observed nanoparticles were spherical in shape. The size and shape of the particles was consistent with the manufacturer's description [50].

Figure 4.2 shows the EDS spectrum of the NP preparation. Peaks were observed for gold (Au) copper (Cu) and sulphur (S) as expected. Other peaks present are bromine (Br), chlorine (Cl), carbon (C), and oxygen (O) and these could be from the preparation media or from the plastic slides on which the particles were imaged.
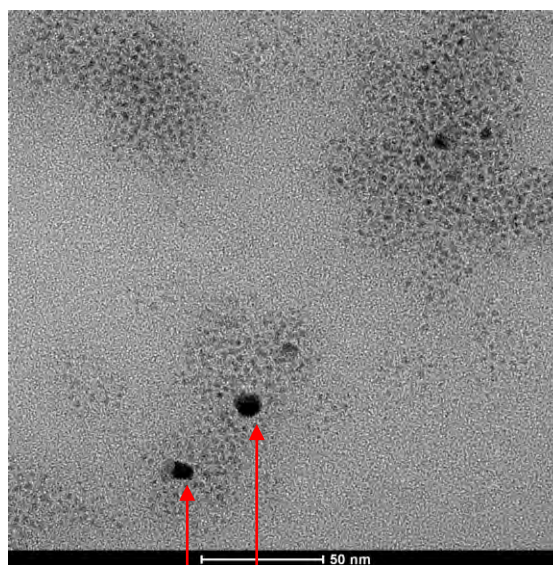
Figure 4.1: TEM images of Au/CuS NPs.Red arrows show clumps of NPs.
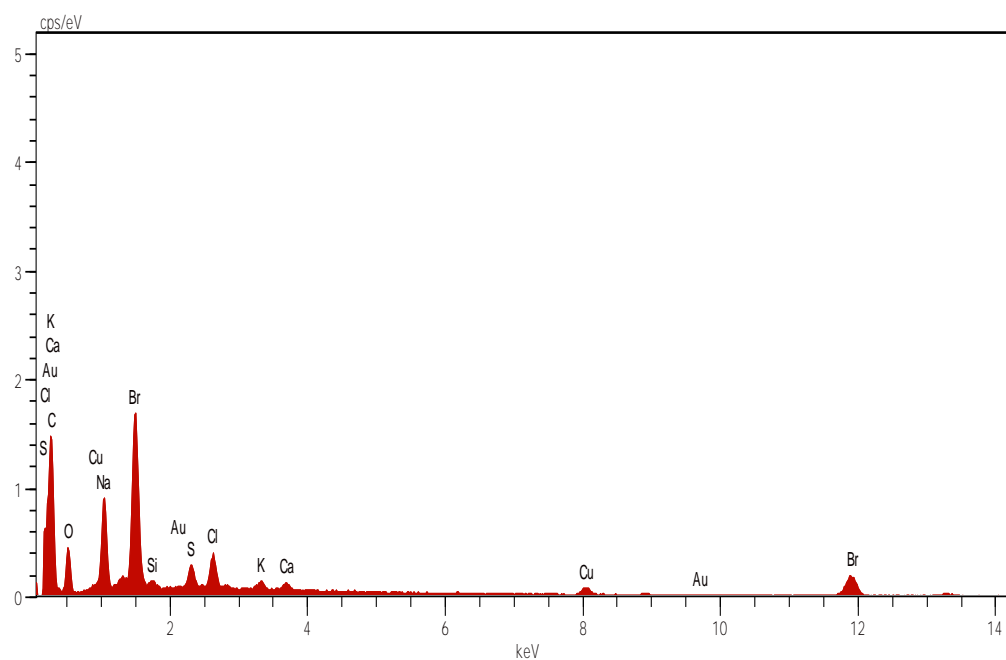
Figure 4.2: EDS spectrum of Au/CuS NPs

4.2 Effect of Au/CuS NPs pre-treatment on the viability of *B. anthacis* spores

Figure 4.3 shows the survival percentage after *B. anthracis* spores were treated with different concentrations of Au/CuS for different durations. *B. anthracis* spores (10^5 in DI-H_2O) were treated with 0.83, 4.15, and 8.3 µM of Au/CuS for 1, 6, and 24 h and then surface plated.. Treatment with 0.83 µM Au/CuS for 1 to 24 h, killed 16 - 20 % of the spores, while treatment with 4.15 µM Au/CuS for1 to 24 h yielded spore killing rate of 17 − 23 %.

Figure 4.3: Effect of [Au/CuS] and treatment duration on *B. anthracis* spores. Initial spore concentration: 10^5 in DI-H_2O.

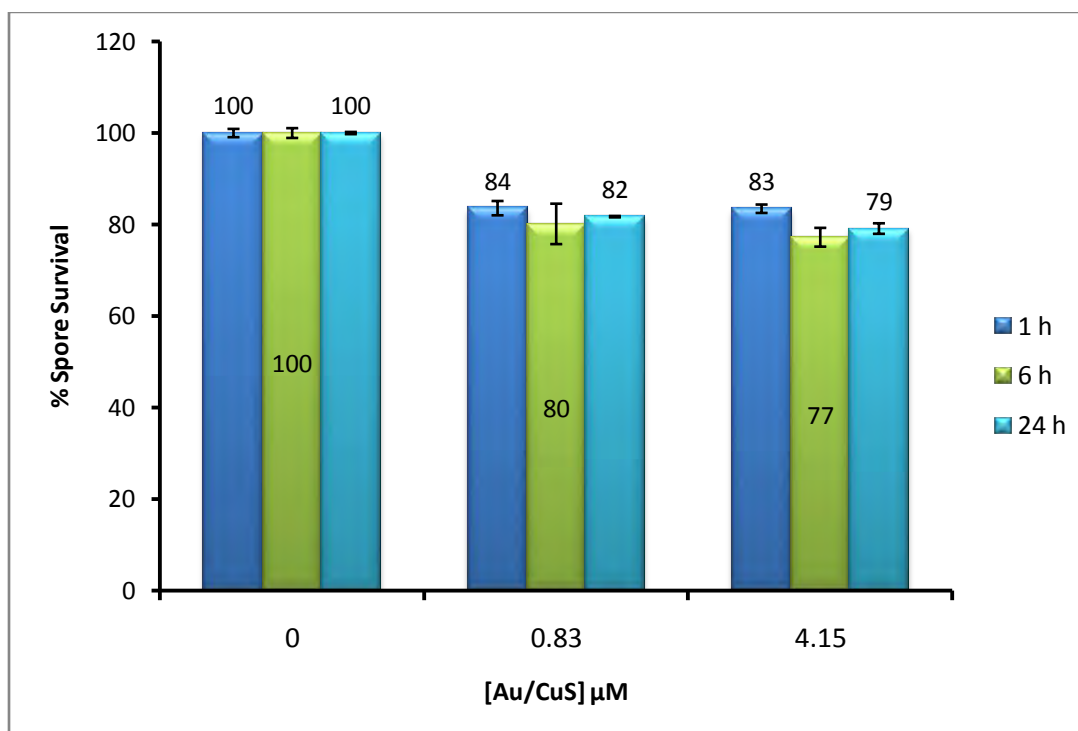Figure 4.4 shows SEM images of Au/CuS (4.15 μM) treated and untreated spores. The absence of any differences in observed spores between the control spores and the treated spores was consistent withthe resultsobtainedfrom the plating experiments. The results indicated that the increasing NP contact time with spores produced no significant increase in the percentage of spores killed by the treatment of various concentrations of NP. Similarly, the increasing concentration of the NPs produced no substantial spore killing effect.

Even though the NPs produced some antimicrobial activity against the spores, they were not very effective, evenat very high concentrations.This is consistent with other reports on the sporicidal activity of some other nanomaterials to bacterial spores. Afewrchich et al. [57] reported that carbon nanotubes (50- 300 μg/mL) were ineffective in killing *B. anthracis* spores even though they effectively killed their cells. They reported that the presence or absence of direct contact between the cells and the nanotubes was responsible for the antimicrobial activity observed [57].

However Stoimenov et al. (2002) reported that metal oxide NPs are very effective in killing *B. subtillis* spores [13]. These conflicting observations on the sporicidal activity of nanomaterials may be due to the variety in the properties of the nanomaterials tested as well as the diversity in the bacterial species. It would be useful to test a single nanomaterial against a variety of bacterial spores to confirm or disprove their sporicidal activity. However, in this case, we found that Au/CuS NPs were ineffective in killing *B.anthracis* spores and that high concentrations exhibited little effect. This could be due to resistance of the hard coat of their endospores to the NP treatment. Hence, the Au/CuS

NPs would not be suitable for killing *B. anthracis* spores especially when one is looking at reducing the cost and increasing the effectiveness of decontamination and also reducing the amount of residual NPs left in the environment after decontamination.
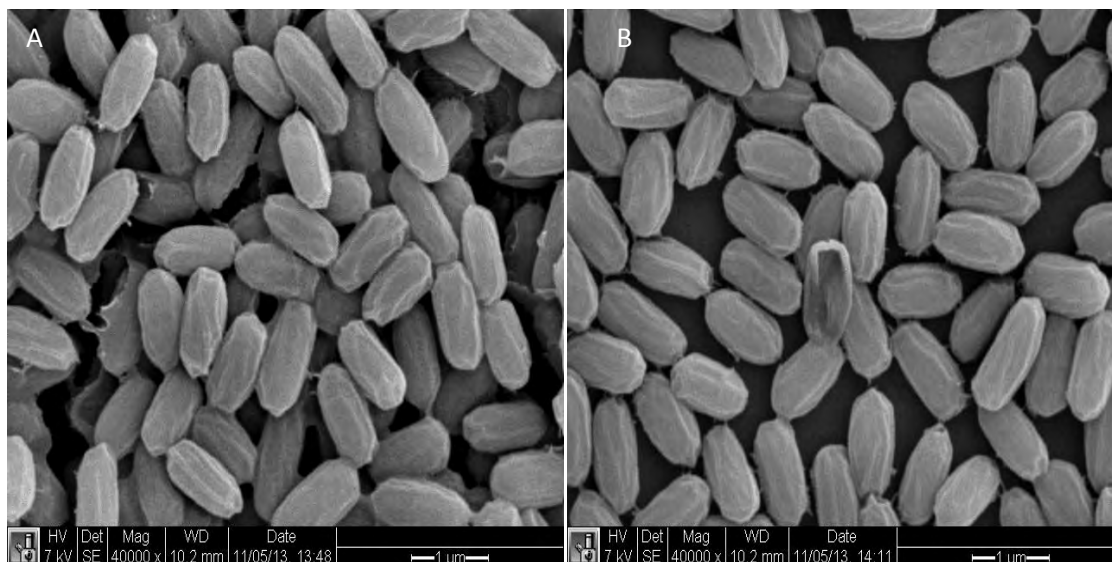

Figure 4.4: SEM images of untreated spores (A) and spores treated with 4.15μM Au/CuS NPs for 1 h(B) .

4.3 Effect of the presence of Au/CuS NPs on the growth of *B. anthracis* spores.

Figures 4.5 and 4.6 below show the outgrowth of spores in nutrient broth after being pre-treated with 0.083, 0.83 and 4.15 μM Au/CuS for 30 min and subsequent growth either in the presence of the NPs (Figure 4.5) or in the absence of NPs (NPs removed immediately following treatment) (Figure 4.6). The controls in both treatments contained no NPs. It is observed from Figure 4.5 that the presence of 4.15 and 0.83 μM Au/CuS completely inhibited spore outgrowth even after 10 h of incubation. But spores treated with 0.083 μM Au/CuSexhibited growth within 6 h of incubation. Thus a concentration dependent

34

inhibition of spore growth was observed when NPs were present in the growth medium. When the NPs were removed after the pretreatment (Figure 4.6: 0.83 µM B) the spores grew after 2 h of incubation which was similar to the control. Meanwhile, the same concentration of NPs, when continuously present in the treatment medium, inhibited spore growth. From these observations, it can be deduced that the continuous presence of the NPs in the medium could be responsible for the inhibition of spore growth, whereas the pretreatment produced no significant spore inhibition or killing.
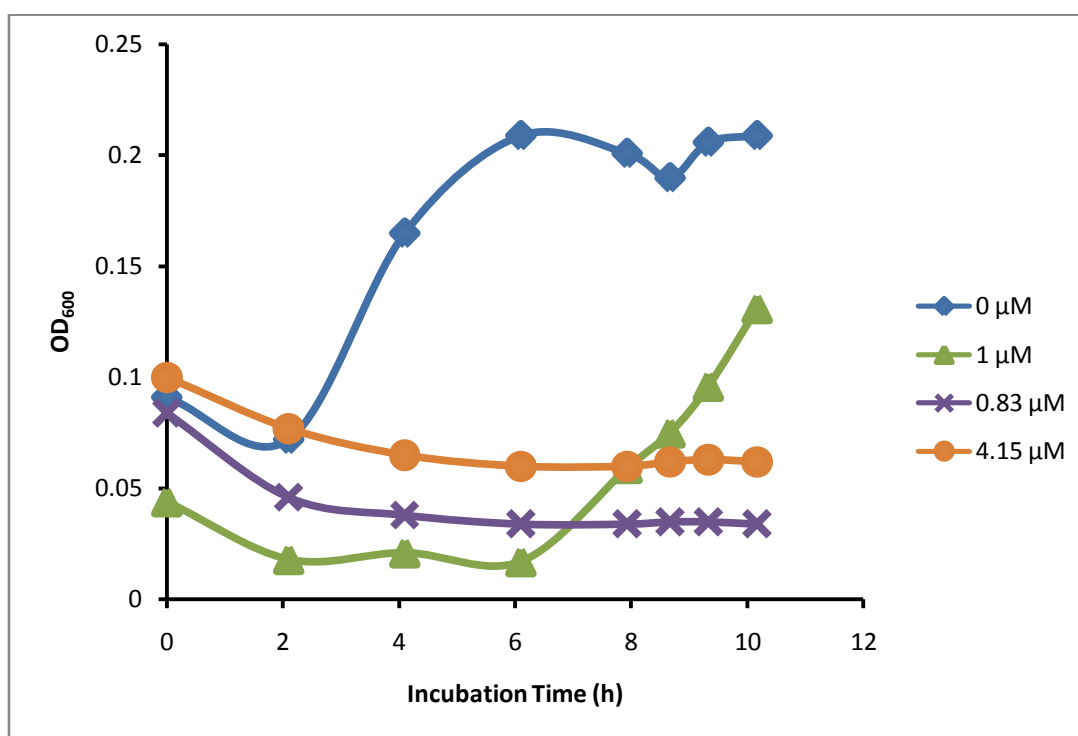

Figure 4.5: Effect of continuous Au/CuS presence on spore outgrowth. Initial spore concentration:10^7, NPs treatment time: 30 min.
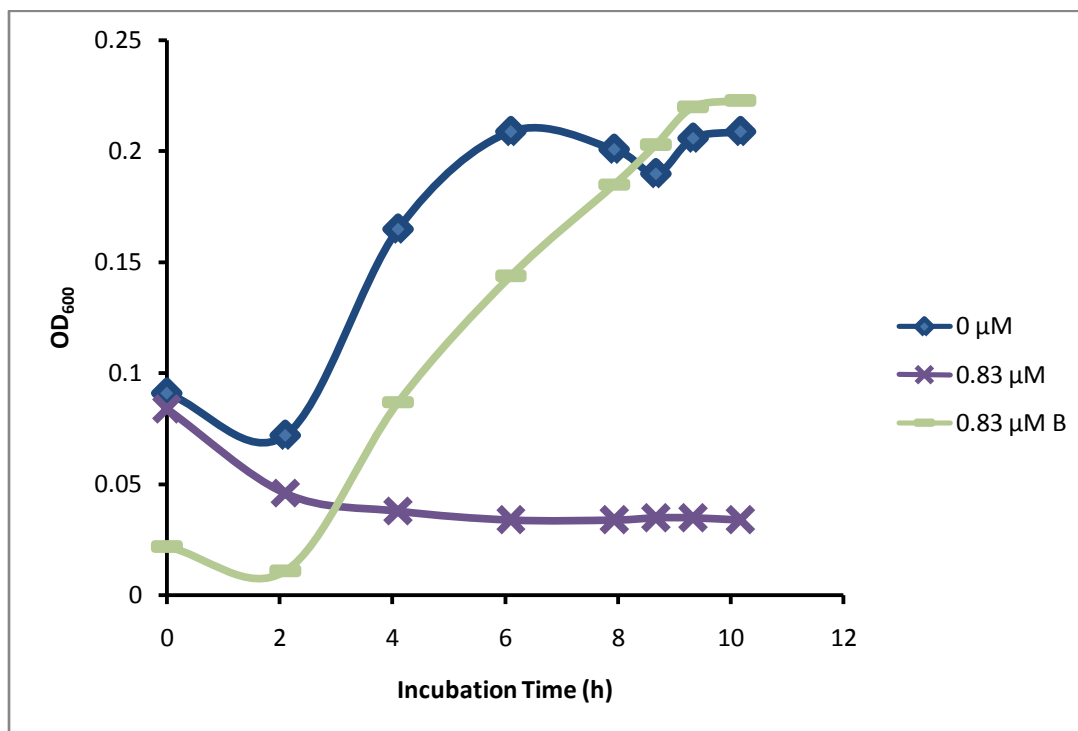
Figure 4.6: Pre-treatment verses continuous incubation of *Bacillus anthracis* spores with Au/CuS NPs. Initial spore concentration: 10^7, NPs treatment time: 30 min. "0.83 µM B" is the growth curve of the sample which the NPs were removed after pretreatment, while "0.83 µM" is the growth curve of the sample which the NPs were not removed after pretreatment.

A possible explanation for this is that NPs remain in solution and kill *B. anthracis* cells as the spores grow into cells. This is similar to the observations of Sloan and Farmsworth[45]. When investigating the antimicrobial effect ofNPs on *E. coli* and *B. anthracis*, they exposed the spores of *B. anthracis* to silver, silver-iron and silver-nickel NPs of different sizes in suspension and plated the bacteria. In their report they explained that the NPs first attached to the spores and then when the spores germinated, the NPs neutralized the emerging vegetative cell, thereby nipping it in the bud.

4.4 Effect of Treatment time and concentration of Au/CuS treatment on inactivation of *B. anthracis* cells.

We then investigated the effect of Au/CuS NPs on the inactivation of *B. anthracis* cells. Figure 4.7 shows the percentage of surviving cells following treatment with different concentrations of Au/CuS NPs on *B. anthracis*cells in DI-H_2O for 0.5, 1, and 3 h. It is observed that the 0.083 μM Au/CuS produced 6, 7 and 12 % cell killing in 0.5, 1 and 3 h, respectively.   Treatment with 0.83 μM Au/CuS for 30 min and 1 h produced a 53% and 100% cell killing effect respectively. When the NPs concentration increased to 4.15 μM, 30 min treatment produced a 100% spore killing effect. Thus the NPs exhibit a concentration and time dependent inactivation of *B. anthracis* cells.
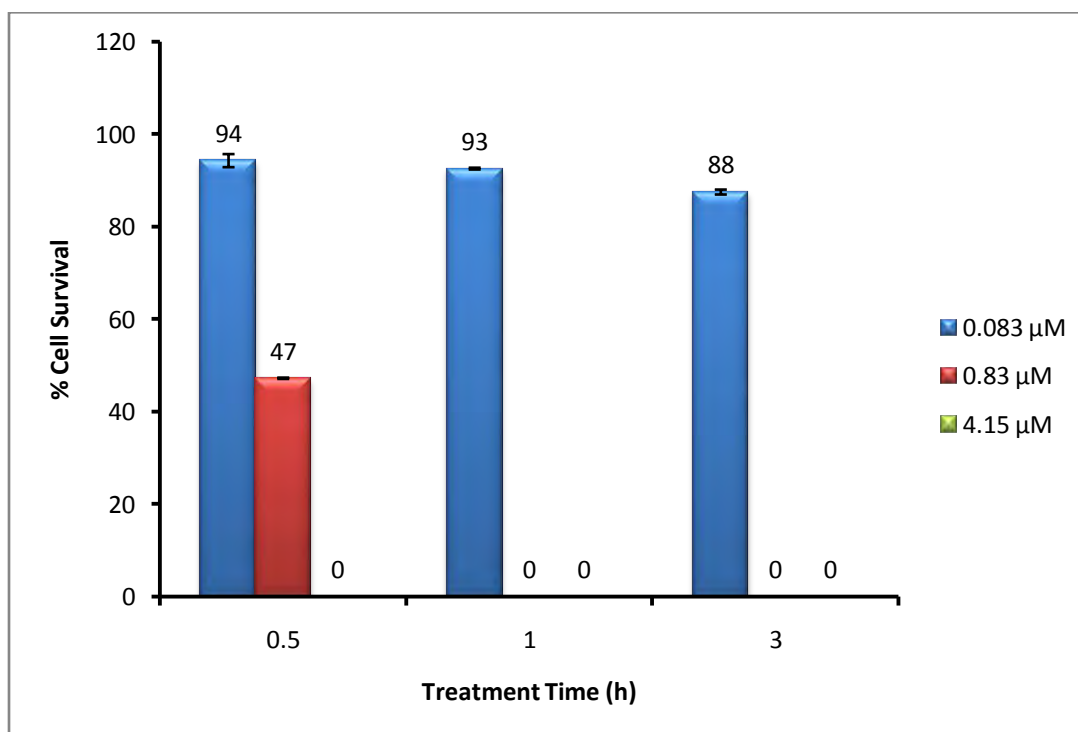

Figure 4.7: Effect of [Au/CuS] and treatment duration on *B. anthracis*cells in DI-H_2O

To confirm the time dependency of the antibacterial activity of the NPs to *B. anthracis* cells, fluorescence images of cells treated with NPs for 10, 20, and 30 min and then stained with live/dead backlight bacteria stain were obtained. Live/dead BacLight bacteria staining kit (Invitrogen, New York) contains two stains: Syto 9 which is a green fluorescence nucleic acid stain and propidium iodide which is a red fluorescence nucleic acid stain. When used alone, Syto 9 stains all bacteria, (dead or alive) green. However, PI penetrates only the dead bacteria which have a disrupted membrane staining them red and thus decreasing the fluorescence of Syto 9 stain. The presence and increase in the number of red cells following increasing treatment duration confirms the time dependency of the NP killing effect.

Figure 4.8 shows the images of *B. anthracis* cells (A) without treatment, and with 10 min (B), 20 min (C), and 30 min (D) treatment with 0.83 µM Au/CuS NPs. The images showed that cells without NP treatment were green (live); bacterial cells treated with Au/CuS for 10 min, showed some cells dead (red) and some live cells (green). An increasing number of dead cells are observed after 20 and 30 min treatments.
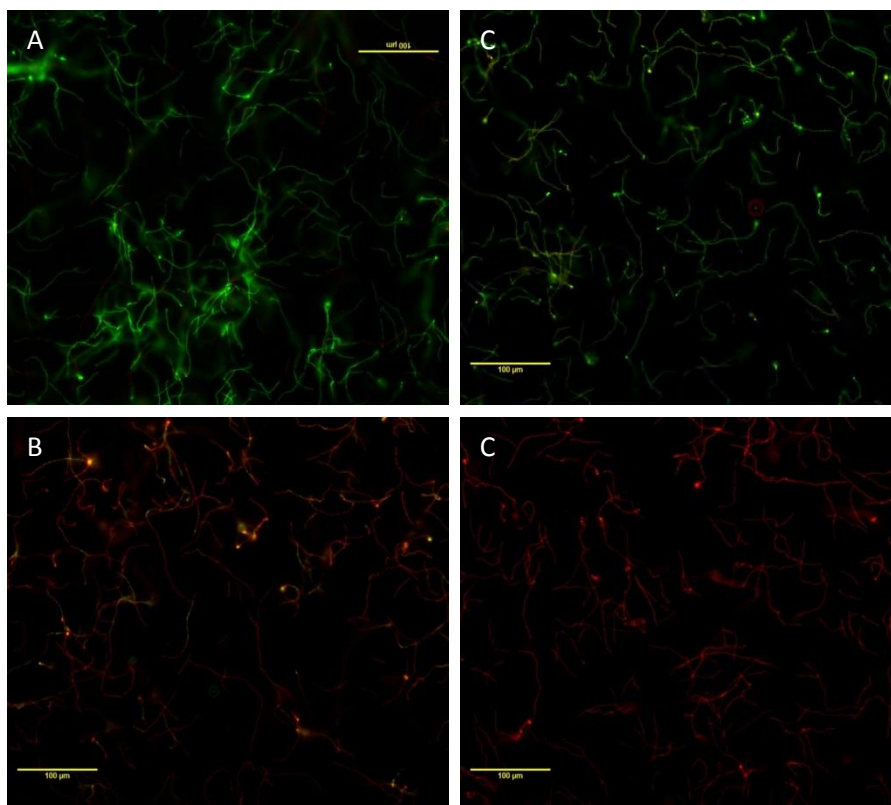
Figure 4.8: Fluorescence images of *B. anthracis*cells treated with 0.83 μM Au/CuSfor 0, 10, 20 and 30 min (A, B, C & D respectively) and stained with live/dead bacteria stain.

4.5 Effect of treatment medium on the antimicrobial activity of Au/CuS to *B. anthracis*cells.

Figure 4.9 shows the percentage of surviving cells after *B. anthracis* cells were treated with different concentrations of Au/CuS NPs for 1 h in three different media (PBS, DI-H_2O and nutrient broth). The results indicated that the 0.083 μM Au/CuS treatment was ineffective in killing *B. anthracis* cells, producing only 3, 6, and 4 % cell kills in PBS, DI-H_2O and nutrient broth respectively; the 0.83 μM Au/CuS treatment killed 83, 100, and 78 % of the cells in the three media respectively, and the 4.15 μM Au/CuSkilled 100

% of the cells in all three media. Thus the treatment of Au/CuS at concentrations of 0.83

and 4.15 μM were effective in killing *B. anthracis* cells regardless of the media (PBS, DI-

H_2O and nutrient broth). The results pointed to the fact that the inactivation of *B.*

anthracis cells was more dependent on the concentration of NPs, and treatment media did

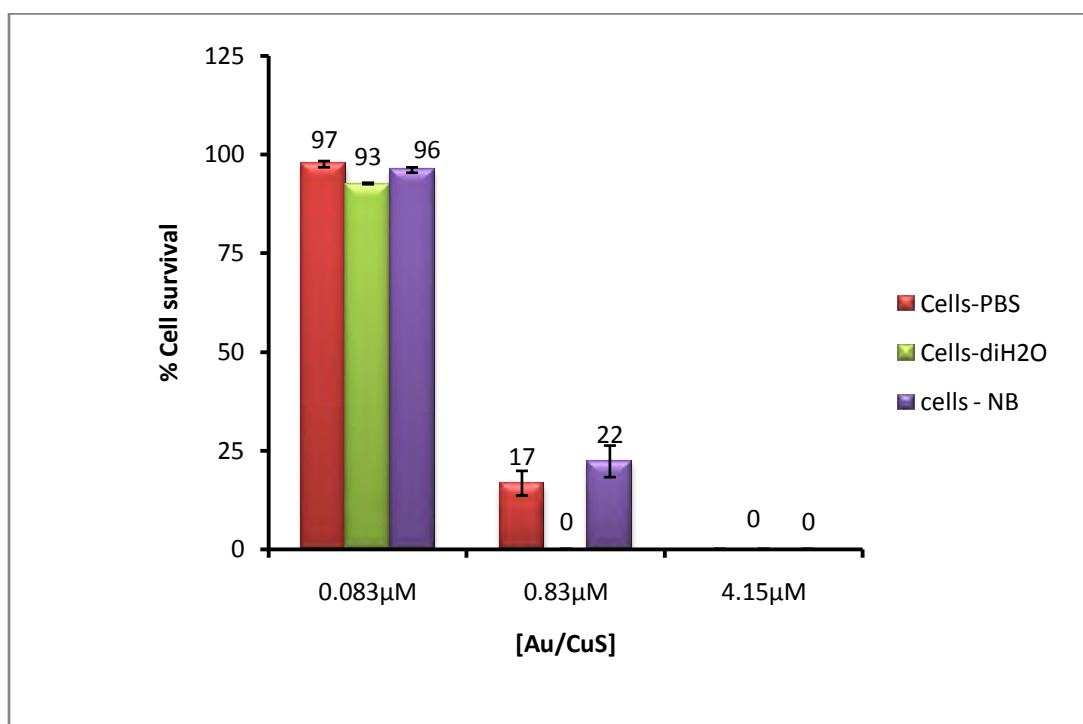not significantly affect the in inactivation efficiency.

Figure 4.9: Buffer effect on Au/CuS NP treatment of *B. anthracis* **cells**

4.6 Possible mechanisms of inhibition of *B. anthracis* cells by Au/CuS NPs.

A number of mechanisms have been proposed for the antimicrobial activity of NPs. To

understand the death of cells treated with Au/CuS NPs and to ascertain the mechanism of

Au/CuS action, Scanning Electron Microscope (SEM), Energy Dispersive X-ray Spectroscopy (EDS) and DNA efflux experiments were performed.

4.6.1 Scanning Electron Microscopy (SEM) of Au/CuS treated cells

Scanning Electron Microscope (SEM) images of treated and untreated cells were obtained. Figure 4.10 shows SEM images of (A) control *B. anthracis* cells, and (B) cells treated with 4.15 µM Au/CuS NPs for 30 min. The control cells showed an intact membrane while the cells treated with NPs had disordered and dispersed membranes. The results revealed that the NPs damaged the cell membranes and eventually caused cell death. This is consistent with the observation reported by Li et al [58],where they investigated the mechanism of silver NPs on *Escherichia coli* cells.In their report, they indicated that silver NPs damaged the cell membrane structure and depressed the activity of some membrane enzymes leading to the death of the cell. Au/CuS seemed to be producing the same effect in their interaction with *B. anthracis* cells.
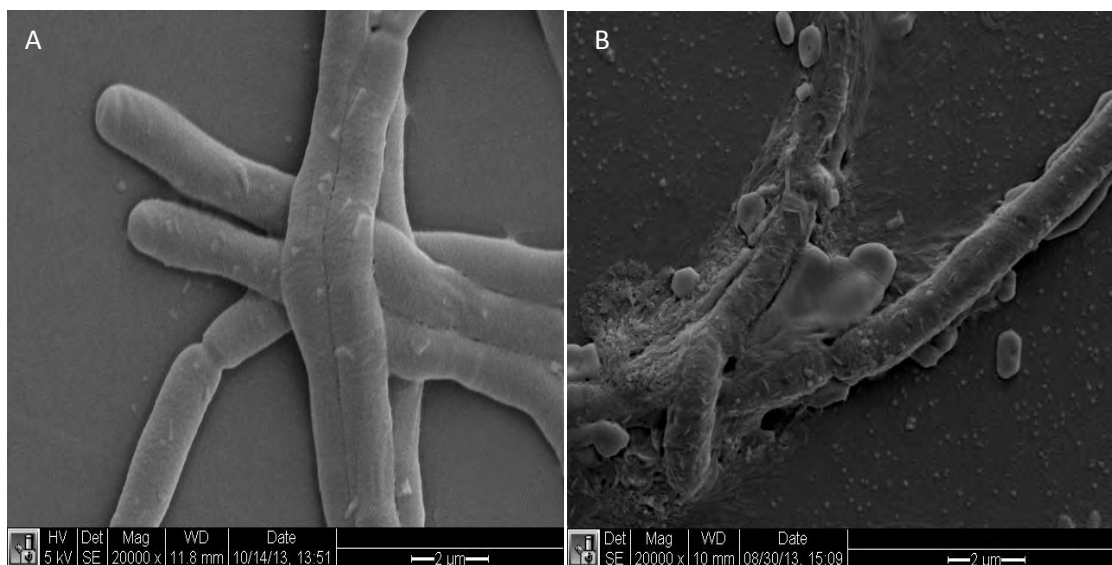
Figure 4.10: SEM images of untreated (A) andAu/CuS NPs treated cells (B).

4.6.2 Energy Dispersive X-ray Spectroscopy (EDS) of Au/CuS treated Cells

To confirm that the Au/CuSNPs were responsible for the death of the cells, the elemental composition of cells, either treated or untreated with NPs was determined using EDS. Figures 4.11 and 4.12 below show the EDS spectrum of the control *B. anthracis* cells and *B. anthracis* cells treated with 4.15 µM Au/CuS NPs respectively. The spectrum from the cells has carbon, oxygen, chlorine, sodium, phosphorus and aluminum (Figure 4.11). In addition to the elements in the EDS spectrum of the control cells, the Au/CuS treated samples have a peak for sulphur and copper (Figure 4.12). These could be coming from the CuS shell of the NP.

It was expected that a peak would be observed for gold. However, this was not the case. This could be due to the adsorption of the x-rays from the gold core by the CuS shell or

by other elements in the specimen. However, the presence of peaks for the copper and the

sulphursuggestedthe presence of the gold copper sulphide NP in the treated sample.
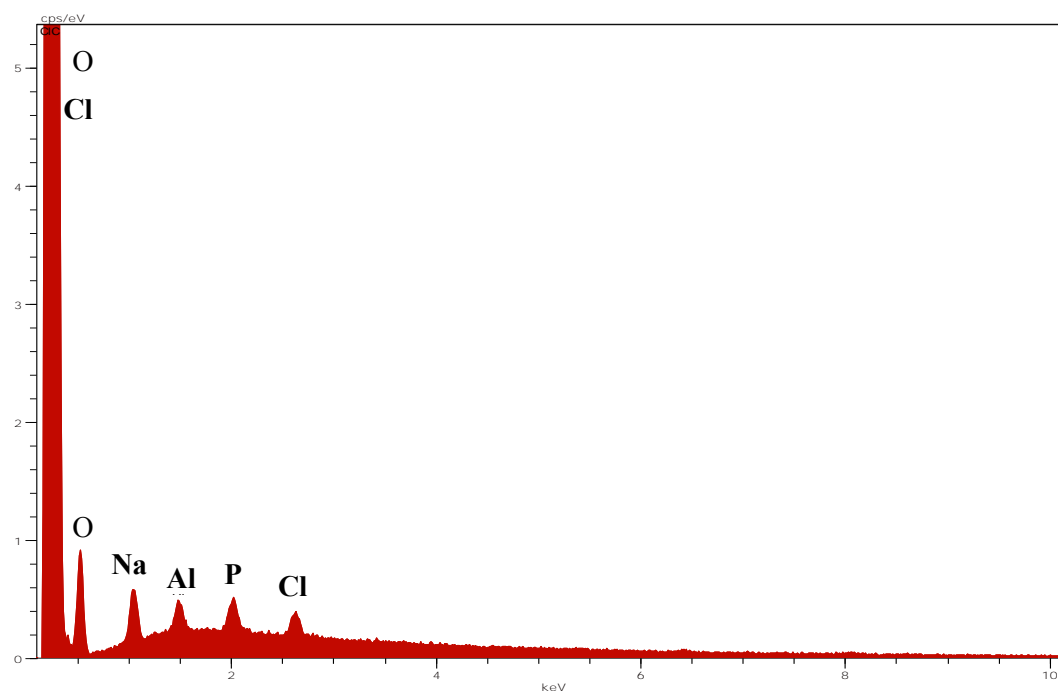
Figure 4.11: EDS spectrum of untreated *B. anthracis*cells
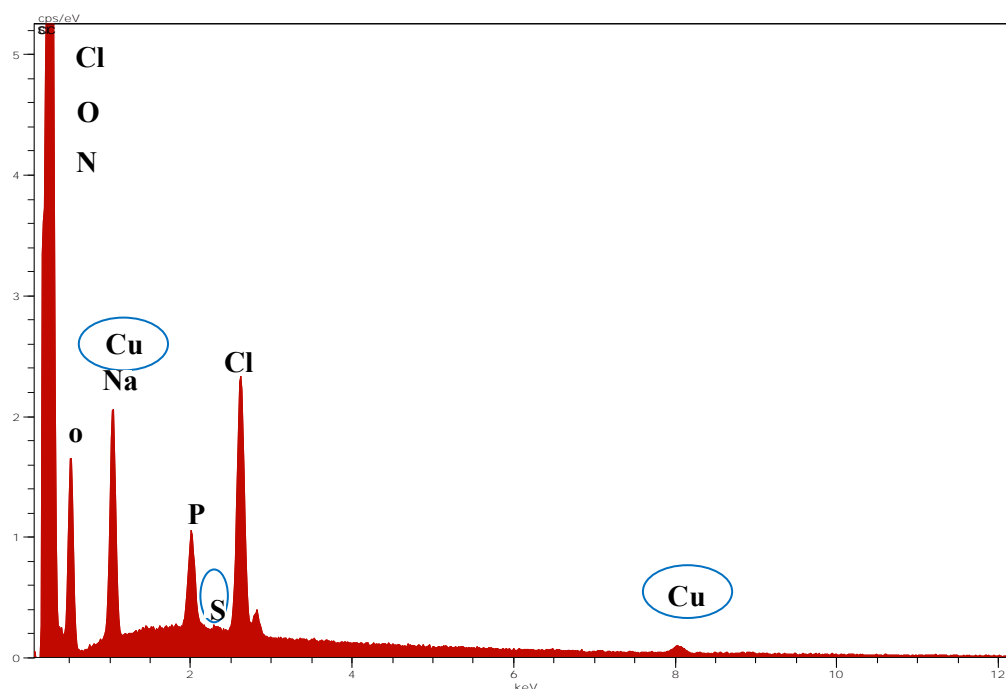
Figure 4.12: EDS spectrum of *B. anthracis* cells treated with 4.15 μM Au/CuS

4.6.3 DNA efflux

To confirm that the NPs damaged the cell membranes of *B. anthracis* and caused the efflux of the cellular components from the treated cells, experiments were conducted to measure the amount of DNA leaked from the cells when cells were treated with 0.83 and 4.15 μM Au/CuS NPs. The results (Figure 4.13) showed that cells treated with 4.15 μM NPs leaked 2.50+/-0.06 μg/mL DNA from the cells while cells treated with 0.83 μM Au/CuS NPs leaked 1.68+/-0.08 μg/mL into the buffer. This confirmed the destruction of cell membranes by the NPs and the efflux of cytosolic contents from the cells. It also further confirmed the concentration dependency of the NP treatment. The insert in Figure

4.13 shows the calibration curve of the salmon sperm DNA used to determine the concentration of the efflux of DNA.
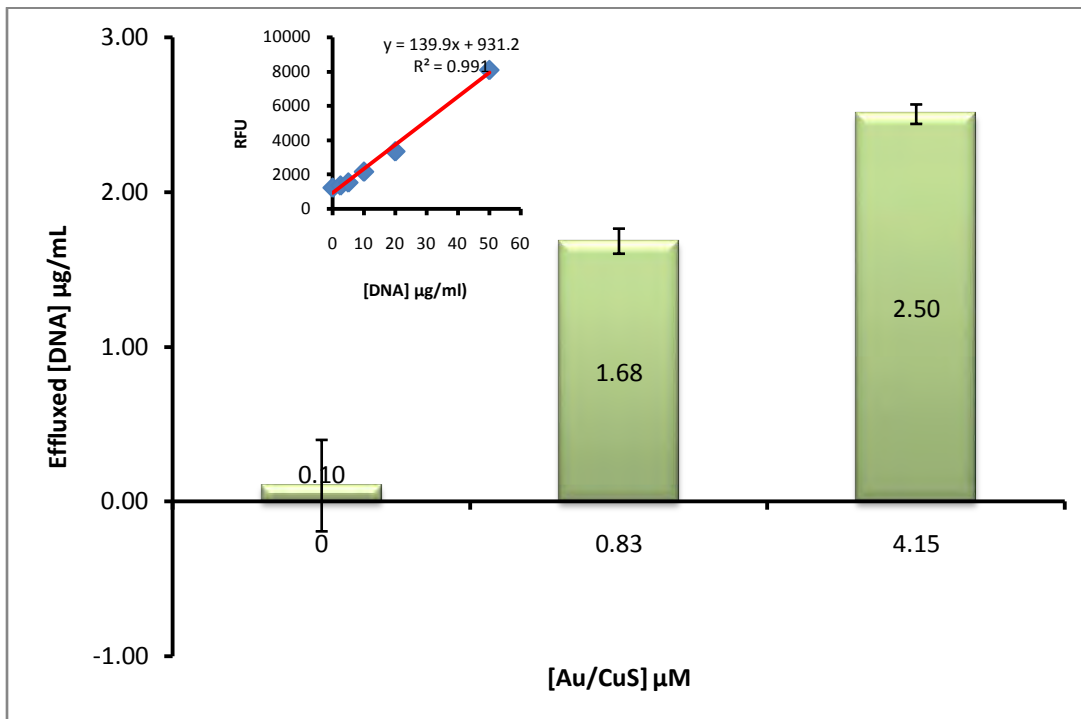
Figure 4.13: Amount of DNA effluxed from *B. anthracis*cells treated by 0.83 – 4.15 μM Au/CuSfor 30 min. Insert: Calibration curve used to determine DNA concentration.

The results of this study showed that the Au/CuS NPs exhibited strong antimicrobial activity against the *B. anthracis* cells. There are reports that electrostatic interactions between negatively charged bacterial cells and positively charged NPs are necessary for the activity of NPs as antibacterial agents [13, 59]. The cell wall is composed mainly of a thick peptidoglycan layer linked to teichoic acid, and gives the cell an overall negative charge. This facilitates its interactions with the positively charged NPs and possibly resulted in the piercing of the membrane by the NPs. This may likely lead to the entry of

substances from the environment resulting in an osmotic imbalance and the consequent rupture of the cells. However, further work is still needed to completely understand the mechanisms of the Au/CuS NPs inactivation of bacterial cells.

CHAPTER **V**

CONCLUSIONS

We investigated the antimicrobial activity of Au/CuS nanoparticles against *B. anthracis* cells and spores. Based on the results obtained, the major findings and conclusions drawn from this research included the following:

1. Au/CuS NPs were not effective in inactivating *B. anthracis* spores (10^5cfu/mL) by pre-treatment with 4.15 µM NPs up to 24 h. However, when the NPs were not removed following the pretreatment and remained in the growth medium, 0.083 µMNPs started to exhibit the inhibition of spore outgrowth and 4.15 µMAu/CuS NPs effectively inhibited spore outgrowth, most likely by NPs inactivating the germinated spores or the outgrown vegetative cells.

2. The Au/CuS NPs exhibited much more effective antimicrobial activity against *B. anthracis* cells than against *B. anthracis* spores. NPs at 4.15 µM inactivated all cells (10^7cfu/mL) in 30 min whiles the same concentration only produced 14% in 24 h.

3. The antimicrobial activity of the nanoparticles to *B. anthracis* cells was concentration and treatment time dependent.

4. SEM imaging provided evidence of cell membrane damage and cell death following treatment with Au/CuS NPs. EDS results confirmed the presence of Cu and S elements on the surface of NPs treated cells.

5. DNA efflux confirmed the destruction of cell membranes by the NPs.

Future directions:

Future experiments on this project would focus on:

1. Confirming the suggested mechanism of nanoparticle destruction of cells and ascertaining if there are other mechanisms not discussed in this work.

2. Investigating if the combination of Au/CuS nanoparticles with other antibacterial agents would enhance its spore killing effect. Also, the surface of the NP could be modified with chemicals that induce spore germination to see if that enhanced its sporicidal activity.

3. The effect of the NP on other bacterial spores and cells.

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